"VITAMIN D RECEPTOR GENE POLYMORPHISMS (VDR) AND STEROID RECEPTOR STATUS IN BREAST CANCER PATIENTS"

SYNOPSIS Submitted In Partial Fulfillment of The Award of Degree of Doctor of Philosophy

In The Department of Biochemistry

SUBMITTED BY ASHOK KUMAR DOGRA

SUPERVISOR

Dr. Archana Prakash, Prof. Dept. of Biochemistry

CO-SUPERVISOR

Dr. Sanjay Gupta, Prof. and Head, Dept. of Biosciences

Dr. Meenu Gupta, Prof, Dept. of Radiation Oncology

Swami Rama Himalayan University
Swami Ram Nagar, Jolly Grant, Dehradun.

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Vitamin D Receptor (VDR) Gene Polymorphisms and Steroid Receptor Status in Breast Cancer Patients

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Mr. Ashok Kumar Dogra
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Subject: Approval of synopsis and allotment of Supervisor for Ph.D.

Departmental Research Committee (DRC) held on 13/08/2019 has approved your synopsis.

In accordance with the recommendations made by the DRC, you are hereby allowed to proceed with research work in your subject area, in the Department of Bioscience under the Dr. Archana Prakash (Supervisor), Dr. Sanjay Gupta & Dr. Meenu Gupta (Co-Supervisor) on the duly approved title given hereunder:

"Vitamin D Receptor (VDR) gene polymorphisms and steroid receptor status in Breast Cancer patients"

Uma Bhardwaj
Dean Research
Research & Ph.D. Cell

Copy to: Dean/Principal/Director/ HOD
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1.0 Introduction

Cancer, is a general term for a wide-range of diseases in which abnormal cells tissues divide uncontrollably and can metastasize to nearby tissues. Breast Cancer and Lung Cancer are the most common cancers globally, affecting a little over 2 million individuals per year. In 2018, out of breast, colorectal and lung cancer, majority (25.4%) of new cases diagnosed with cancer was contributed by breast cancer alone (World Cancer Research Fund International, 2019) and an estimated 0.6 million i.e., approximately 15% of women died from breast cancer. Though breast cancer rates are higher among female from West and other developed regions, its incidence is increasing rapidly among developing regions due to lifestyle modification, urbanization and increased life expectancy. Early detection, such as diagnosis and screening for breast cancer is essential for better outcomes and survival however, limited resource setting among low and middle income setting and lack of awareness among women on its signs and symptoms makes early diagnosis of breast cancer a challenge(WHO, 2019a, 2019b). Being a preventable disease, every women should be aware of factors which has the ability to increase the likelihood of developing breast cancer such as smoking cigarettes, consuming alcohols, being overweight, lack of exercise, exposure to chemicals and radiation, not breastfeeding, taking hormones and eating unhealthy diet which could be modified by choosing a healthy lifestyle. However, other risk factors such as age, gender, reproductive history, race or ethnicity, personal and family history of cancer, especially breast cancer, and genetics cannot be modified. Most emerging risk factor for breast cancer among women in both developed and developing countries are lower levels of Vitamin D(Breastcancer.org, 2019; Centers for Disease Control and Prevention, 2018).

Several population based studies which report on the incidence and mortality rate among women with breast cancer, states that women living in geographic regions with greater biologically effective solar UV-B (280–315 nm) exposure have lower incidence and mortality rates from breast cancer (Anderson et al., 2011; Grant, 2012; Bilinski et al., 2014; Grigalavicius et al., 2016). UV light is essential for the conversion of 7-dehydrocholesterol to pre-vitamin D3 in the cutaneous and subsequent isomerization to Vitamin D3. Biologically active form, steroid hormone 1,25-dihydroxyvitamin D (calcitriol) is essential to regulate a large number of biologic pathways such as inhibition of cell proliferation, angiogenesis, metastasis, invasion and stimulation of cell differentiation and death (de La Puente-Yagüe et al., 2018). Its deficiency, can contribute to the pathogenesis of some cancers, autoimmune

diseases, multiple sclerosis, cardiovascular disease, infectious disease and type 2 diabetes mellitus (T2DM)(Wacker & Holick, 2013).

Vitamin D Receptor (VDR), a member of the steroid hormone nuclear receptor family, plays an important role in the bio-functioning of calcitriol. Apart from regulating expression of numerous genes involved in calcium and phosphate homeostasis, they promote cell differentiation and inhibit proliferation of certain cells exhibiting cancer prevention properties(Wang et al., 2012). VDR is located in cells of major organs of the body like liver, mammary glands, kidneys, parathyroid glands, heart, bone, intestine, pancreas, prostate, colon and vascular cells(Bikle, 2000). A study by Zhalehjoo et al., 2017) concluded that mRNA expression of CYP27B1(1-alpha hydroxylase) was downregulated and CYP24A1 (24-hydroxylase) upregulated in the malignant breast tissues suggesting dysregulation of the vitamin D signaling and metabolic pathways during cancer formation in breast cells impairing anti-cancer activity of vitamin D.

Since, epithelial cells of mammary glands have the same enzyme 1-alpha hydroxylase as renals, the effects of vitamin D on breast cancer cells is reasonable. Vitamin D is key for normal mammary gland growth and development. Calcitriol, play a major role in opposing estrogen driven proliferation and stimulating differentiation of mammary epithelial cells through VDR gene expression regulation. Several evidence based studies demonstrated that calcitriol plays key role in inhibiting estrogen based cell proliferation and angiogenesis in normal and malignant breast cells and inducing cell differentiation and death in both normal and malignant breast tissues through intervention with VDR(Santos-Martínez et al., 2014),(Giammanco et al., 2015). CYP27B1 mediated 1-alpha hydroxylase in the cells of mammary gland, regulate the development of breast cells (de La Puente-Yagüe et al., 2018). and the regulation of VDR expression is dynamically regulated during different reproductive cycle such as puberty, pregnancy, lactation and weaning for proper glandular development(de La Puente-Yagüe et al., 2018).

VDR gene located on chromosomal locus 12q13-14 has several common allelic variants. There are many different type of single nucleotide polymorphisms (SNPs) reported in VDR (Ruan et al., 2015) of which, four most common polymorphic forms found within the Breast Cancer-VDR gene are rs2228570 (FokI), rs1544410 (BsmI), rs7975232 (ApaI) and rs731236 (TaqI) (Chauhan, 2017).

The VDR gene and its polymorphism involved in breast cancer risk is given under Table 1.

Table 1: VDR gene and its polymorphism

Name of the Enzyme	**VDR gene polymorphism**
rs7975232(Apa-1)	Polymorphism in intron 8 of the enzyme results in T (variant "A") changing into G (variant "a")
rs1544410(Bsm-1)	Polymorphism in intron 8 of the enzyme results in A (variant "b") changing into G (variant "B")
rs731236 (Taq-1)	Polymorphism in exon 9 of the enzyme results in T (variant "T") changing to C (variant "t")
rs2228570 (Fok-1)	Start codon polymorphism in exon 2 of the enzyme results in T (variant "f") changing to C (variant "F")

Source: Adopted From Cieślińska et al., (2018)

Polymorphism of VDR genes have shown to correlate with breast cancer susceptibility and it can influence ones dietary or pharmaceutical therapeutic interventions(Welsh et al., 2003). VDR gene polymorphism leads to dysfunctioning of calcitriol. According to Li et al.(2012), VDR gene polymorphism could lead to significant steroid receptor dysfunction, affecting formation and function of calcitriol.

Serum and tissue analysis of estrogen and progesterone receptor status is increasingly becoming a standard of care for patients diagnosed with breast cancer(Ramteke et al., 2016). Its significance in the development of breast cancer was reported over the last four decades, when it was observed that radiolabeled estrogens was in abdundance in the estrogen-driven target organs of both animal and human breast cancer. From previous literatures, it is evident that approximately 50-70% of breast cancer cases are estrogen receptor positive (ER+). Additionally, 50% of ER+ tumors also have progesterone receptor positive (PR+) status as ER is involved in the regulation of gene transcription encoding the PR in the mammary gland. Since, this finding, it become clear that mammary epithelial cells and breast cancer are dependent upon estrogen and/or progesterone for proliferation and that this effect is mediated through ER and/or PR. Undoubtedly, ER and PR are overexpressed in malignant breast tissue(Elizabeth & Hammond, 2018). Therefore, PR expression results in indirect ER activation(Finlay-Schultz et al., 2017). Immunohistochemical (IHC) analysis are more accurate than biochemical assays in assessing ER status and prediction of mortality risk among breast cancer patients. Followed by IHC assessment, hormonal therapy is initiated to

control estrogen production and suppress its action. Most of the hormonal therapy drugs used for breast cancer either lower estrogen levels or block ER in malignant breast tissues(Society, 2019). Of different drugs, Aromatase inhibitors (AIs) are currently first line drugs used to treat hormone driven breast cancer (ER+) in post-menopausal breast cancer cases as they possess the ability to prevent aromatase enzyme activity and hence estrogen production. However, such hormone therapy may not help breast cancer patients whose tumors are hormone receptor-negative(Bleach & McIlroy, 2018).

Along with ER and PR, accruing evidence suggests a role for Androgen Receptor (AR) expression in the breast cancer cases. AR expression is associated with good patient outcome and its expression in breast cancer tissues depends upon the serum estrogen and progesterone levels. Several experimental studies have reported that AR exhibits its protective role by inhibiting the ER+ gene transcription, however, other studies report conflicting results at high levels of circulating androgens(Giovannelli et al., 2018). Due to its tumor suppressive action against hormone driven breast cancer's (ER+ and /or PR+), therapeutic drugs which can up-regulate (agonists), down-regulate (antagonists) and inhibitor androgen synthesis are under clinical investigation (Bleach & McIlroy, 2018), however, for treatment using AR, circulatory levels of AR and AR status in mammary glands should be assessed periodically(Bronte et al., 2018).

Hence, to understand the epidemiology, factors influencing the levels of Vitamin D in breast cancer and association between VDR gene polymorphism and steroid receptors such as ER, PR and AR status among breast cancer patients, a literature review was conducted using original articles, review papers, evidence based systematic review and meta-analysis published in reputed English medical journal between 2012-2019.

2.0 Literature Review

Study by Bellanger et al.(2018), globally, assessed the impact of countries economic development on the prevention strategies of breast cancer. According to them, over 1.6 million cases of breast cancer and 0.5 million breast cancer deaths has occurred during 2012 of which 60% of breast cancer deaths has occurred from high income and upper middle income countries. Though the incidence of breast cancer was higher among high income countries, occurrence of breast cancer is spirally among low income countries, particularly, among women less than 50 years of age. Key reasons for increased incidence of breast cancer

along with other cancers could be due to adaptation of Western lifestyle (chewing tobacco, eating unhealthy food, sedentary life etc) and increased life expectancy narrowing the gap of breast cancer age between developed and developing countries(Hadi et al., 2018).

A systematic review da Costa Vieira et al.(2017) based on 45 research papers from developing countries evaluating different breast cancer screening strategies and indicators report that, self and clinical examination of breast was primary diagnosis method when mammogram was not feasible. However majority of women in the developing countries were unaware of self or clinical breast examination. Moreover, limited resource settings make early diagnosis of breast cancer difficult. Thus, lack of awareness and accessibility to public mammogram services results in only 0.5% of mammography diagnostic rates among developing countries. Hence, the review recommends national programs and policies to educate and encourage women on breast cancer, self-diagnosis of breast cancer, appropriate age range for mammography diagnosis and availability of public mammography diagnostic facility to reduce mortality risk associated with breast cancer.

Several epidemiological studies and meta-analysis has been conducted to assess the changeable and non-modifiable risk factors linked with breast cancer genesis. According to a study by Maas et al.(2016) non-changeable risk factors such as single nucleotide polymorphism (SNP), family history, menstrual and reproductive status increased the risk of breast cancer, similar to changeable risk factors such as increased body mass index, smoking, drinking and use of menopausal hormone therapy. A meta-analysis conducted by Nindrea et al., (2017), observed that parity followed by over-weight and use of oral contraceptives increased the risk of breast cancer whereas non-modifiable risk factors such as family history, age and menopausal status increase the incidence of breast cancer among women in South-East Asia.

According to Mohr et al.(2011), serum calcidiol level of 47 ng/ml is associated with 50% reduction in breast cancer prognosis. Circulatory levels of 25-hydroxyvitamin D (calcidiol), precursor to active form of Vitamin D reflects the amount of biosynthesized and dietary vitamin D status (Fuhrman et al., 2013). Although there is no standards established on the ideal levels of serum calcidiol, from literatures it is evident that levels less than 20 ng per millilitre (ng/mL) and above 150 ng/mL are consider hypo and hyper-vitaminosis D. Hence, it can be debated that serum levels of calcidiol between 30-60 ng/mL as ideal. Several

evidence based studies have confirmed an inverse association between breast cancer risk and serum levels of calcidiol(McDonnell et al., 2018; Estébanez et al., 2018).

Out of different factors affecting biosynthesis of vitamin D, presence of type 2 diabetes mellitus (T2DM), pigmentation of the skin, sunscreen use, menopause and estrogen deficiency play crucial role. A quantitative non-linear dose-related meta-analysis of 9 prospective studies assessing the link between circulating calcidiol levels and breast cancer risk among menopause women(Bauer et al., 2013), reported that postmenopausal breast cancer risk reduce with calcidiol levels of 27-35ng/mL and a 5 ng/mL rise in calcidiol was linked to 12% reduction in breast cancer occurence. However, calcidiol levels below 27 ng/mL and above 35ng/mL show flat association with breast cancer risk.

Several epidemiological studies have confirmed strong association between impairment in the pathway of Vitamin D mechanism, VDR gene polymorphism and breast cancer risk and cancer risk prognosis (Iqbal et al., 2015; Guo et al., 2015; Reimers et al., 2015; Abd-Elsalam et al., 2015). Similarly, many case control studies and meta-analysis have been conducted to identify specific gene polymorphisms in VDR that involved in breast cancer risks(Rai et al., 2017). For example, a meta-analysis conducted between Dec 2015-January 2017 on various medical databases showed that polymorphism of vitamin D receptor i.e, Apa1, Bsm1, Poly (A) and Fok1 are linked with the occurrence of breast cancer, while Taq1, Cdx2 and Bgl1 do not indicate association. It was further concluded that, Fok1 polymorphism results in postmenopausal breast cancer when compared to premenopausal breast cancer(Iqbal & Khan, 2017).

A systematic review, which tried to find association between specific VDR gene polymorphisms and individual malignancies, reported that, one VDR gene polymorphism may have different effect based on the type or location of malignancy, or it can only increase or decrease cancer aggressiveness. According to the review Bsm1, Taq1 are strongly associated with cancer prognosis VDR polymorphism, whereas Bsm1, Fok1 are strongly associated with VDR polymorphism, other least associated VDR polymorphism includes Apa1 and poly (A). Also, it was reported that, Fok1 was most commonly associated with different types of malignancies such as prostate, skin, colo-rectal, ovary and bladder(Köstner et al., 2009).

A study by Mishra et al.,(2013) reported that f allele of Fok 1 was linked with the incidence of breast cancer risk among Afro-American population. However VDR gene polymorphism and ApaI alleles (Aa or aa) was more associated with breast cancer among Hispanics or Latinas. Five year disease-free-survival (DFS) of breast cancer with VDR-Fok1 *FF* genotype was poor. In contrary to the above findings, a meta-analysis conducted among Caucasian women on gene polymorphism of vitamin D receptor and breast cancer risk reported no substantial association between homozygote, heterozygote, recessive and dominant Fok1, Bsm1, Apa1 and Taq1 genotype and breast cancer risk(Yang et al., 2014). However, a newly, case-control study, which assessed VDR gene polymorphism and steroid receptor status of breast cancer affected Saudi women, reported that, the frequency of ff genotype increased in breast cancer group than controls and carriers of f allele are at the risk of developing breast cancer. Also, the study concluded that there exists a positive relationship between Fok1 gene polymorphism and estrogen receptor status (Nemenqani et al., 2015).

In the Indian setting, breast cancer found among Indian population have lower rates of positive ER and PR, which usually occur among younger population, often large during diagnosis and high grade when compared to their counterparts. According to (Kaul et al., 2011), IHC staining of malignant breast tissues of women living in Himalayan region of India was found to be lower in positive ER and PR.

A systematic review and meta-analysis by (Sandhu et al., 2016), concluded that triple negative breast cancer, i.e, cancer which tests negative for estrogen receptor (ER), progesterone receptor (PgR), and the human epidermal growth factor receptor 2 (HER-2/neu) is highly prevalence among Indian women when compared to western population. These negative breast cancer are more severe with higher relapse or recurrence rate when compared to single and/or double positive breast cancers such as ER+ alone or ER+ and PR positive breast cancers respectively (Singhai et al., 2011). Hence, analysis of receptor status is crucial as it helps to determine the prognosis of the disease and treatment regimen to be followed(Iqbal & Buch, 2016). Since, triple negative breast cancers cannot be treated using hormonal therapy and anti-HER-2 targeted therapies like ER/PR positive breast cancer's, hence, current treatment options for triple negative breast cancer's include Neoadjuvant chemotherapy, chemotherapy or radiotherapy in conjunction with surgery. A detailed treatment approaches available for triple negative breast cancer is discussed by Wahba and El-Hadaad(2015).

3.0 Research Aims and Objectives

The proposed aim of the study is to assess VDR gene polymorphisms and steroid receptor status among Breast Cancer patients.

Objective 1: To identify the different VDR gene polymorphisms among patients presenting Breast Cancer.

Objective 2: To identify the allele and genotype of individual VDR gene polymorphisms involved in the occurrence and prognosis of breast cancer.

Objective 3: To represent odds ratio (OR) of Estrogen Receptor, Progesterone Receptor and Androgen Receptor status in normal and malignant breast tissues.

4.0 Research Questions

RQ1: What serum levels of Vitamin D is associated with increased Breast Cancer risk?

RQ2: What are the distribution of VDR gene polymorphisms among patients with Breast Cancer?

RQ3: Which allele and genotype of VDR gene polymorphism is involved in increasing the risk of breast cancer?

RQ4: What percentage of positive and negative Estrogen Receptor, Progesterone Receptor and Androgen Receptor status are found in malignant and normal breast tissue?

RQ5: Is the levels of Androgen Receptor and Estrogen Receptor status in breast cancer inversely associated?

5.0 Research Methodology

Study Setting

An institution-based study will be conducted at Swami rama himalayan University**,** a teaching-tertiary care hospital, located in swami ram nagar, Jolly Grant, Dehradun, Uttarakhand, India. The above location was chosen primarily because as a paucity of data is available related to the study topic. Despite the above, being a tertiary care hospital dealing with Breast Cancer

patients, collection of data will not be difficult. Due to financial and time constraints, the study period shall be restricted to two years.

Study Design

It is a matched case-control study in which newly detected breast cancer patients and control group will be included in the study. The population characteristics include women population who is aged between 30 to 70 years belonging to Indian nationality.

From nMaster2.0 software, sample size will be estimated considering coordinated case-control study (1:1) matching. Supposing that proportion of controls epxosed is 50% taking a level of significance 5% with power equal to 90% in order so as to detect a two-fold increase risk. Minimal required number of discordant pairs is 110.

The study shall include both pre and post-menopausal women however excludes those with previous history of breast cancer, those undergoing cancer medication or hormonal therapy, those under Vitamin D supplementation or previously under Vitamin D supplementation in the past 6 months, those belonging to ethnicity other than India and finally all pregnant and lactating women.

Patient's demographic information such as age at detection, pathologic primary tumour size, histologic subtype (grade), lymph node status, number of lymph nodes involved and Reproductive or menopausal status shall be collected.

Post which, individual participant's blood sample shall be collected and collated for DNA extraction. PCR-Restriction Fragment Length Polymorphism shall be done to identify the allele, genotype of individual VDR gene polymorphism. From record, receptor status of steroid hormones estrogen, progesterone and androgen should be taken. Genotyping and Vitamin D quantification will be done according to DNA Kit.

All samples will be collected prior to chemotherapy. For purpose of the analysis, patients will be categorized in two ways, firstly based on age and secondly based on the stage of the tumour.

All the data collected shall be stored in MS Excel and examine using statistical package for the social sciences (SPSS) software. Descriptive analysis will be put forward for the demographic and clinical characteristics. The genotype and allele frequencies shall be evaluated by chi-square test to compare observed and expected genotypic distributions. One-

way ANOVA will be used to test the connection between vitamin D concentration and genotype. The association between breast cancer and polymorphisms of vitamin D receptor will be assessed first with χ 2 test. Odds ratio (OR) shall be calculated for having progression of breast cancer for individual genotypes which will be estimated using logistic regression models. A ($P < 0.05$) will be considered as statistically significant(Chakraborty et al., 2016; Ahmed et al., 2019).

As the study involves human participation, the study should be conducted in accordance with the guidelines set by the Institutional Ethical Committee and approval to be obtained. Table 2:

Study Design	Matched Case-Control Study
Study Period	Two years
Study Location	Single institutional study conducted at Swami Rama Himalayan University**,** a teaching- tertiary care hospital located in swami ram nagar, Jolly Grant, Dehradun, Uttarakhand, India.
Inclusion Criteria	- newly diagnosed cases of breast cancer - pre and postmenopausal women
Exclusion Criteria	- Personal history of breast cancer - Under therapy - Under Vitamin D supplementation - Under hormonal therapy - Pregnant - Lactating - Ethnicity other than Indian
Clinical data	Sample blood will be collected from participants (Breast cancer cases and control group) and DNA shall be extracted
Demographic information	- age, - tumour size, - grade, - lymph node involvement, - Reproductive status
Molecular studies data	Genotypes of VDR gene FokI, Bsm1, Apa1, Poly (A), Cdx2, Bgl1, and Taq1 will be identified by (PCR-RFLP). Odds ratio (OR) of having progression of breast cancer for having these genotypes shall be calculated.
Steroid Receptor Status	Assessment of ER, PR and AR status by immunohistochemical (IHC) analysis
Ethical committee approval	The study should be conducted in accordance with the guidelines set by the Institutional Ethical Committee
Data Analysis	Microsoft Excel and SPSS software.
Statistical Analysis	For statistical analysis chi-square test will be used. Also for statistically significant, $P < 0.05$ shall be considered.

6.0 Significance of the Study

The findings of this study will be useful for researchers, medical practitioners, individual stakeholders who are interested to know the type of VDR gene polymorphism and its allele and genotype involved in increasing the appearance and prognosis of breast cancer as its treatment involves consideration for VDR, SNPs and serum of Vitamin D. The information collated from this research work can contribute to improve health and healthcare of patients and population affected with breast cancer or having high prognosis for breast cancer occurrence.

7.0 Limitations of the Study

Our major limitation of the study is being a single institutional study conducted at a teaching- tertiary care hospital, we cannot include a large cohort to provide more conclusive results. However, based on the study outcome, a large multi-centre, randomized control trial study can be conducted in near future.

8.0 Summary

Breast Cancer are most common cancer among women in both developed and developing countries. Early detection, such as diagnosis and screening for breast cancer is essential for better outcomes and survival however, limited resource setting among low and middle income setting and lack of awareness among women on its signs and symptoms makes early diagnosis of breast cancer a challenge. Being a preventable disease, every women should be aware of changeable risk factors associated with breast cancer, however, other risk factors such as gender-based, age, personal and family background of breast cancer, race or ethnicity, reproductive history and genetics cannot be changed or modified. Out of different risk factors, the most emerging factor is lower levels of serum Vitamin D which is essential for normal development of mammary glands.

Genetic variability of VDR or VDR gene polymorphism have shown to correlate with breast cancer susceptibility. This is because VDR gene polymorphism leads to dysfunctioning and formation of calcitriol have a vital role in opposing estrogen driven proliferation and stimulating differentiation of mammary epithelial cells through VDR gene expression regulation.

Though many case control studies and meta-analysis have been conducted to identify specific allele and genotype of individual VDR gene polymorphisms associated in breast

cancer, the results published are often debatable due to variation in geographical, racial and ethnic distributions, along with inclusion of sample size etc. Hence, inconsistent results in finding the association between VDR gene polymorphism and breast cancer risk necessitates the need for more epidemiological studies in large cohorts. Hence, the study aims to assess VDR gene polymorphisms and steroid receptor status among breast cancer patients in India.

Despite few unavoidable limitations, findings of this research work shall contribute to improve health and healthcare of patients and population affected with breast cancer and those having high prognosis for disease.

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